Tufts Synthetic Biology Summer Notebook

iGEM 2015

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July 2, 2015: An aliquot of purified pHis1522-aTcdB vector was acquired from Dr. Xingmin Sun from the Tufts Vet School. NIH-recommended simple letter format MTA forms were signed by both parties. (aTcdB = atoxic *Clostridium difficile* toxin B)

July 6, 2015: The pHis1522-aTcdB was diluted 1:10 and 1ul was transformed into JM109 *E. coli.* Cells were plated under ampicillin selection and incubated at 37C overnight.

July 7, 2015: Two colonies of JM109-pHis1522-aTcdB were picked to inoculate overnight liquid cultures.

July 8, 2015: Both cultures were retrieved from the incubator, but left benchtop.

July 9, 2015: Around 4 mL of each culture was used for a miniprep using the MoBio UltraClean® Standard Mini Plasmid Prep Kit. DNA concentration of each sample was determined via nanodrop (135.5ng/ul and 170.2 ng/ul).

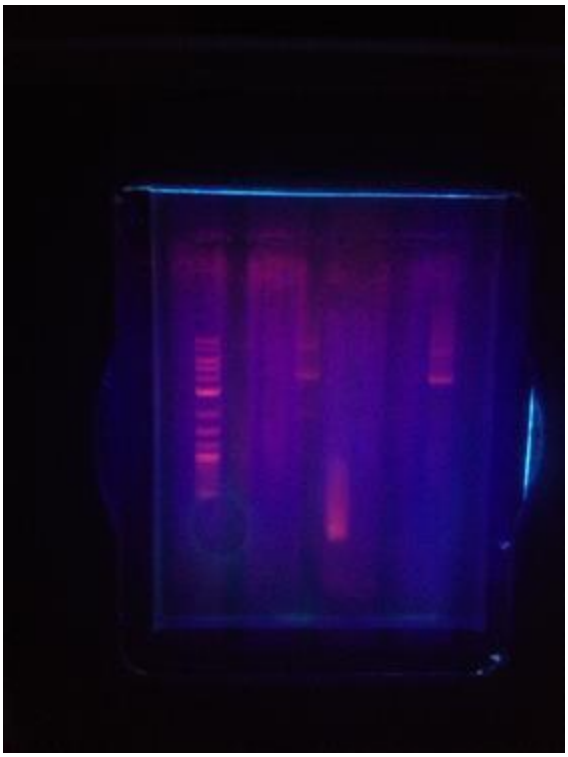
July 13, 2015: Primers were ordered for the pHis1522-aTcdB and pHsp70-Cas9 vectors. The primers were designed to install 21 extra bp at the ends, to be used as homologous overlaps for Gibson assembly of the NLS-tagged Cas9 upstream of the aTcdB gene.

The pHsp70-Cas9 vector arrived from Addgene as a bacterial stab. It was streaked out on an ampicillin plate and left to grow overnight.

July 14, 2015: Three colonies harboring the pHsp70-Cas9 vector were used to inoculate overnight liquid cultures.

July 15, 2015: The three cultures were miniprepped and the sample concentrations were checked by nanodrop (32.9 ng/ul, 33.3 ng/ul, 118.3 ng/ul).

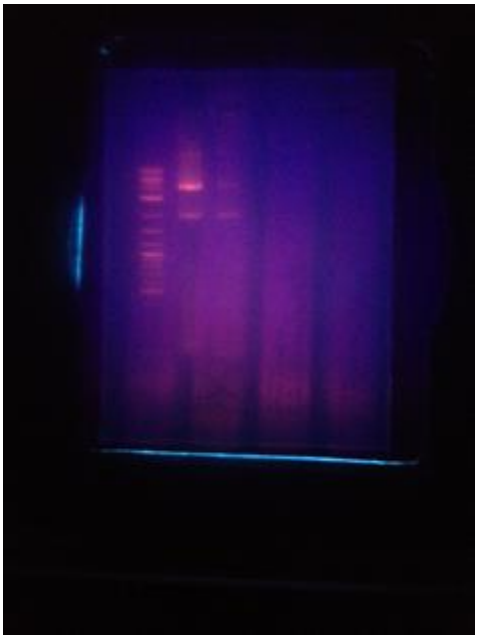
July 16, 2015: The NLS-tagged Cas9 was amplified from each miniprep template using the NEB Q5 HF Polymerase. This was tried with annealing temperatures of 64C and 68C.



1. Ladder
2. Cas9 A 64C
3. Cas9 B 64C
4. Cas9 C 64C
5. Cas9 A 68C
6. Cas9 A 68C (duplicate)
7. Cas9 B 68C
8. Cas9 C 68C

Products were seen only with the 'Cas9 C' template. The desired 4242 bp product was seen, as well as an undesired product around ~2000 bp. The 4242 bp band was gel purified with the MoBio DNA Purification Kit.

July 20, 2015: Now using the gel purified band as a template, amplification of the NLS-tagged Cas9 was retried. Again, the secondary product around 2000 bp was seen.



1. Ladder
2. Cas9 C 64C
3. Cas9 C 68C

The desired ~4 kb band was gel purified with the MoBio DNA Purification Kit.

July 21, 2015: A PCR to linearize the pHis1522-aTcdB vector was performed. A picture was not taken, but the desired ~14kb product was seen and purified both by gel extraction or PCR cleanup (37.4 ng/ul and 30.4 ng/ul).

July 22, 2015: Cultures of JM109 *E. coli* were started from glycerol stocks and incubated overnight.

July 23, 2015: Competent *Bacillus megaterium* were obtained from Dr. Xingmin Sun from the Tufts Vet School.

The overnight cultures were used to seed 100 mL cultures that grew about six hour until OD_{600} of approximately 0.6. The cells were then made competent via resuspension in 0.1M $CaCl_2$ and stored at -80C.

July 27, 2015: Assembly of the purified NLS-tagged Cas9 product and the linearized pHis1522-aTcdB vector was attempted via NEB HiFi assembly. The reaction mixture was transformed into JM109 cells with heat shock.

July 28, 2015: No colonies were seen, even after 24 hours.

August 3, 2015: The HiFi reaction to assemble the NLS-tagged Cas9 and linearized pHis1522-aTcdB was redone and transformed again into JM109 *E. coli* as well as NEB 5-alpha Competent *E. coli* (undiluted and 1:10 dilutions were tried).

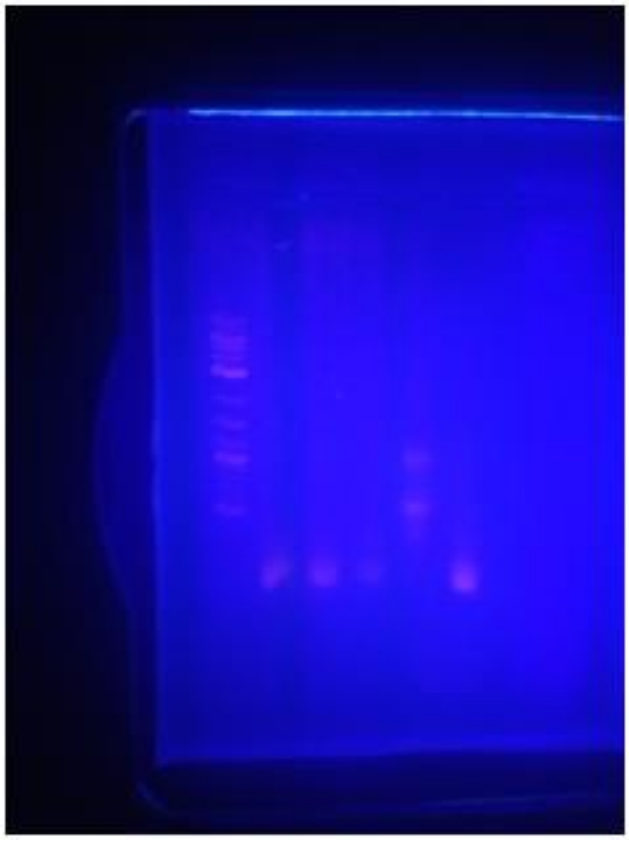
August 4, 2015: Around 100 colonies were seen on the 1:10 5-alpha plate. Eight colonies were picked and grown in liquid media overnight.

August 5, 2015: Around 4 mL of each culture was miniprepped with the MoBio kit. In all isolates, DNA concentration was only around 2-3 ng/ul.

August 7, 2015: The minipreps were retried, but concentrations remained very low.

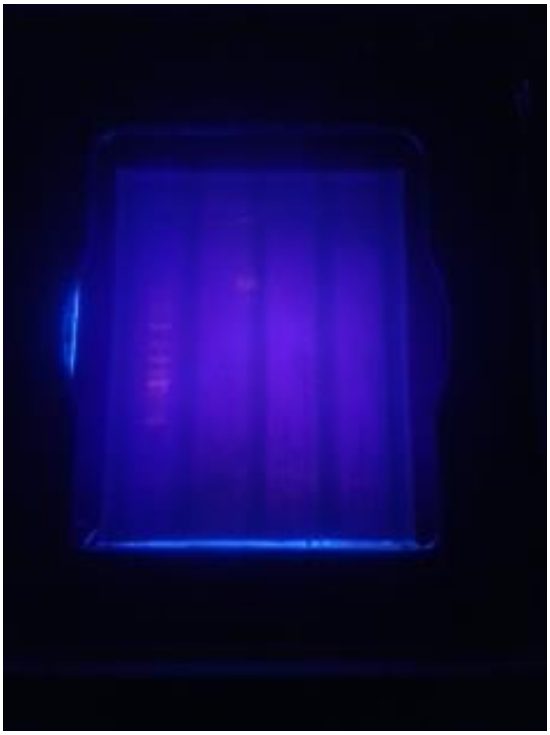
August 10, 2015: Suspecting that the nearly 19kb vector may be too large for silica columns, plasmid isolation via ethanol precipitation was attempted. Yields were substantially better (most better than 50 ng/ul).

A PCR to amplify the NLS-tagged Cas9 was performed on these minipreps to determine whether Cas9 was actually inserted.



Although not visible because of a low res camera, there is a faint ~4kb band in sample 3, suggesting Cas9 was inserted successfully.

August 11, 2015: To further confirm the desired plasmid was isolated, 10 ul of each miniprep was run directly on a gel.



Only sample 3 appears to have any plasmid DNA.

August 13, 2015: pSB1C3-J04450 was solubilized from the 2015 iGEM Distribution plates and transformed into JM109 *E. coli.*

August 14, 2015: Several colonies were observed. Three were picked and grown overnight.

August 16, 2015: The cultures were restarted and again grown overnight.

August 17, 2015: Minipreps were performed on the three cultures. Yields were generally good. A gBlock to synthesize the guide RNA was ordered from IDT. It consists of a T7 promoter, dual oppositely-oriented BbsI cut sites, the gRNA scaffold, and a terminator. It is based largely on this construct: http://flycrispr.molbio.wisc.edu/protocols/gRNA It also has the Biobrick RFC10 prefix and suffix.

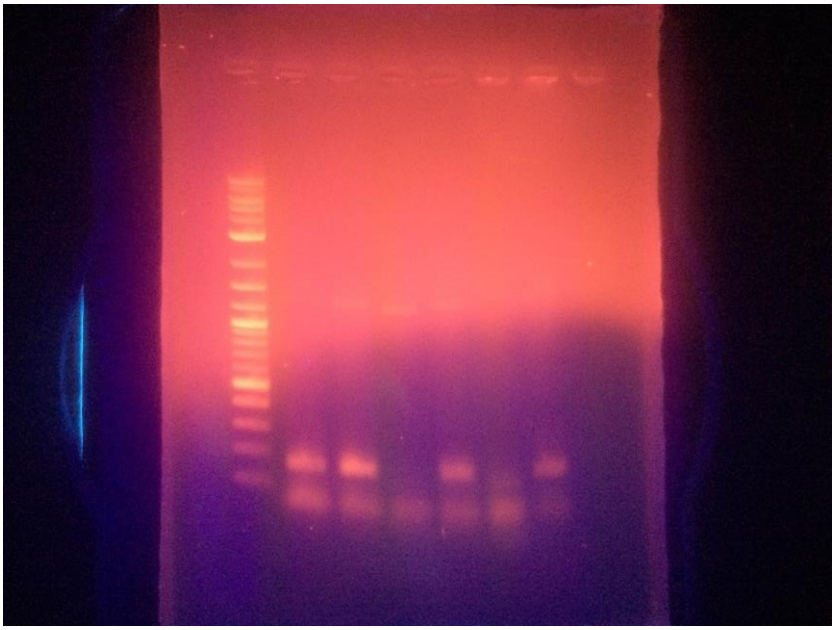
August 26, 2015: A PCR reaction was performed on the T7-BbsI-gRNA gBlock. The desired 171 bp product was seen on a gel. The reaction was purified.

August 27, 2015: 500 ng of the purified T7-BbsI-gRNA PCR product and 500 ng of pSB1C3-J004450 were separately digested with EcoRI and PstI. The pSB1C3-J04450 was dephosphorylated with NEB rSAP following digestion. Both reactions were purified with the MoBio DNA Purification Kit. The two constructs were ligated with T4 DNA ligase and transformed into both JM109 and NEB 5-alpha *E. coli.*

August 28, 2015: Many colonies were observed. Some lacking RFP were grown overnight.

August 29, 2015: Minipreps were performed on all cultures. Yields were generally good.

August 30, 2015: PCR reactions were run on these minipreps to confirm that the T7-BbsI-gRNA construct was successfully inserted.



Samples 1, 2, 4, and 6 seem to have the desired 171 bp band.

September 8, 2015: T7-BbsI-gRNA-pSB1C3 minipreps 1,2,4, and 6 were presumed to have the insert and sent out for sequencing by Eurofins Operon.

September 10, 2015: Sequencing confirms proper insertion of the T7-BbsI-gRNA cassette. This construct is to be submitted as our first Biobrick.

September 12, 2015: The T7-BbsI-gRNA-pSB1C3 plasmid was digested with BbsI and then dephosphorylated with rSAP. A pair of oligos meant to target the pET15b plasmid were annealed and phosphorylated with NEB T4 Polynucleotide kinase. The reaction were purified, ligated together with T4 Ligase, and transformed into NEB 5-alpha *E. coli.*

September 13, 2015: Many colonies were observed. Sixteen were picked and grown overnight.

September 14, 2014: All cultures were miniprepped.

September 15, 2015: All sixteen minipreps were sent out for sequencing to determine whether the annealed oligos were actually inserted.

September 17, 2015: Sequencing results were returned. None of the samples contained the desired insert. The reads suggest the vector was either not cut or re-ligated to itself.